Processing of foreign proteins synthesized using baculovirus vectors in insect cells

3

3.1 INTRODUCTION

The widespread use and success of the baculovirus system makes it impractical to provide an exhaustive survey of all the foreign proteins synthesized in insect cells. Instead, it is our aim to consider the co- and post-translational processing events that have been demonstrated to occur in insect cells and to illustrate these with selected examples.

The insect cell appears capable of undertaking many of the processing events that are required for the formation of biologically active, heterologous proteins. The following processing events will be discussed: glycosylation; phosphorylation; fatty acid acylation; amidation; proteolytic processing, including signal-peptide cleavage; cellular targeting and secretion; tertiary and quaternary structure formation. Post-transcriptional processing will also be mentioned very briefly, although at the present time there is little data on this topic. Several recent review articles will provide the reader with more comprehensive surveys of genes that have been expressed using baculovirus vectors (Fraser, 1989; Maeda, 1989a; Miller, 1988; Atkinson *et al.*, 1990a; Bishop and Possee, 1990; Possee *et al.*, 1990; Vlak and Keus, 1990; Luckow, 1990).

The amounts of protein synthesized by recombinant baculoviruses in insect cells vary from a yield equivalent to that of polyhedrin (approximately 500 mg/litre cells), to relatively low yields of less than 1 mg/litre cells. Unfortunately, it is not yet possible to predict with any confidence how well a particular protein will be synthesized,

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Table 3.1 Examples of glycoproteins expressed using baculovirus vectors

Protein	*Species/virus*	*Membrane-targeted (MT) Secreted (S)*	*Reference*
Virus examples			
env-gp85	Avian leukaemia virus	MT[1]	Noteborn *et al.* (1990)
Spike gp	Bovine corona virus	S	Yoo *et al.* (1991)
Haemagglutinin	Fowl-plague virus	MT[1,2]	Kuroda *et al.* (1986)
Surface antigen	Hepatitis B virus	S[1,2]	Kang *et al.* (1987) Takehara *et al.* (1988) Lanford *et al.* (1989)
Glycoprotein D	Herpes simplex virus (type 1)	MT[1,2]	Krishna *et al.* (1989)
gp160	Human immuno-deficiency virus	MT[1,2]	Rusche *et al.* (1987) Hu *et al.* (1987)
Haemagglutinin	Human influenza virus	MT	Possee (1986) Kuroda *et al.* (1989) Kuroda *et al.* (1990)
Fusion glycoprotein	Human parainfluenza virus	MT[1,2]	Ray *et al.* (1989)
F glycoprotein	Human respiratory syncytial virus	MT[1,2]	Wathen *et al.* (1989)
Haemagglutinin	Japanese encephalitis virus	MT	Matsuura *et al.* (1989)
Membrane fusion and env glycoprotein	Measles virus	MT	Vialard *et al.* (1990)
Peplomer gp (E2)	Murine coronavirus (JHM)	MT[1]	Yoden *et al.* (1989)
HN proteins	Parainfluenza virus (type 3)	MT	Van Wyke Coelingh *et al.* (1987)
G protein	Rabies virus	MT[1,2]	Préhaud *et al.* (1989)
Glycoprotein	Vesicular stomatitis virus	MT	Bailey *et al.* (1989)
E and NS1	Yellow fever virus	–	Desprès *et al.* (1991)
Non-virus examples			
Diuretic hormone	*M. sexta* (tobacco hornworm)	S[1]	Maeda (1989b)
Juvenile hormone esterase	*Heliothis virescens* (tobacco budworm)	S[1]	Hammock *et al.* (1990)
Acid β-glucosidase	Human	MT	Grabowski *et al.* (1989)
β-adrenergic receptor	Human	–	George *et al.* (1989)
CD4 receptor	Human	MT[1]	Webb *et al.* (1989)
EGF receptor	Human	MT[1]	Greenfield *et al.* (1988)
Glucocerebrosidase	Human	S[1]	Bergh *et al.* (1990) Martin *et al.* (1988)
Haptoglobin	Human	S[1]	Heinderyckx *et al.* (1989)

Protein	*Species/virus*	*Membrane -targeted(MT) Secreted(S)*	*Reference*
Immunoglobulin Heavy chain (γ-1)	Human	S[1]	Hasemann and Capra (1990)
Immunoglobulin Light chain (91A3)	Human	S[1]	Hasemann and Capra (1990)
Insulin receptor	Human	MT	Herrera *et al.* (1988) Paul *et al.* (1990)
β-interferon	Human	S[1]	Smith *et al.* (1983)
Myelin-associated glycoprotein	Human	S[1]	Johnson *et al.* (1989)
Plasminogen	Human	S[1]	Davidson *et al.* (1990) Whitefleet-Smith *et al.* (1989)
Chimeric plasminogen activators (PA)	Human	S	Devlin *et al.* (1989)
Poliovirus receptor	Human	–	Kaplan *et al.* (1990)
Tissue-type PA	Human	S[1]	Jarvis and Summers (1989)
Transferrin receptor	Human	MT[1]	Domingo and Trowbridge (1988)
Urokinase-type PA	Human	S	King *et al.* (1991b)
$GABA_A$ receptor	Bovine	MT	Atkinson *et al.* (1991)
Phaseolin	*Phaselolus vulgaris* (French bean)	S[1]	Bustos *et al.* (1988)
Ricin B-chain	*Ricinus communis* (Castor bean)	S[1]	Piatak *et al.* (1988)

[1] Antigenic.
[2] Elicited neutralizing antibodies.

although it appears that yields of membrane-associated glycoproteins tend to be on the low side. We hope that the summary in this chapter will provide some useful guidelines as to what may be expected when expressing a new foreign gene.

3.2 GLYCOSYLATION

Many biologically active glycoproteins have been synthesized in insect cells and some examples are listed in Table 3.1. The most common and thoroughly investigated type of glycosylation is the N-linked process that occurs in the endoplasmic reticulum of both mammalian and insect cells. N-linked glycosylation is initiated

by the addition of an oligosaccharide to an asparagine residue (consensus recognition site, Asp-X-Ser/Thr) and is thought to be mediated via a phospholipid carrier (reviewed by Kornfield and Kornfield, 1985; Parekh *et al.*, 1989).

In mammalian and insect cells, N-linked glycosylation may be inhibited by treating cells with tunicamycin. Comparison of mammalian cell- and insect cell-derived glycoproteins, however, has revealed several differences in the nature of the added oligosaccharide side-chains. Mammalian cells extensively trim and then modify the core oligosaccharide in terminal glycosylation events. These events usually result in the addition of fucose, glucosamine-galactose and sialic acid residues to form complex, branched oligosaccharides. Insect cells appear to lack, or have only very low levels, of the necessary fucose, galactose and sialic acid transferases. They appear to lack the ability to process the core oligosaccharide (Butters and Hughes, 1981; Butters *et al.*, 1981). Furthermore, in insect cells the core oligosaccharide has been shown to contain a high proportion of mannose residues. Recombinant glycoproteins synthesized in insect cells may, therefore, be readily detected by radiolabelling with [^{3}H] or [^{14}C]mannose, or by binding to mannose-specific lectins such as concanavalin A. From the few detailed studies performed on recombinant glycoproteins produced by baculoviruses in insect cells, it would appear that about 50% of the core oligosaccharides are trimmed to GlcNAc2Man3, with the remainder having a variable number of mannose residues (Kuroda *et al.*, 1990).

One consequence of these differences in glycosylation is that recombinant proteins in insect cells have a smaller apparent molecular weight, after analysis by PAGE, than their authentic counterparts in the normal host cell. For example, the avian leukaemia virus (ALV) envelope glycoprotein (env gp) has an apparent molecular weight of 85 kDa, whereas the insect cell-expressed protein has molecular weights ranging from 45 to 65 kDa (Noteborn *et al.*, 1990).

Baculovirus-expressed recombinant proteins are sensitive to endo H, endo F and N-glycanase, which remove immature high-mannose type oligosaccharides. The treatment of insect cell-derived recombinant proteins with these enzymes (or treatment of infected-cells with tunicamycin) results in proteins that have the same molecular weight as similarly treated proteins derived from the normal host cell. For example, when the ALV env gp85 was synthesized in the presence of tunicamycin, both the avian and insect cell-derived protein had a molecular weight of 45 kDa (Noteborn *et al.*, 1990). These results have been complemented by a study on the role played by glycosylation in the secretion of tissue plasminogen activator (t-PA) from insect cells (Jarvis *et al.*, 1990b). In this study, treatment

of recombinant virus-infected cells with tunicamycin inhibited secretion of t-PA. However, treatment of cells with inhibitors of N-linked oligosaccharide processing (castanospermine or *N*-methyldeoxynojirimycin) did not inhibit t-PA secretion. These results suggested that addition of N-linked oligosaccharides, but not their subsequent processing, was necessary for t-PA secretion from insect cells.

The first evidence that limited processing of the core oligosaccharides of recombinant glycoproteins can occur in insect cells has been provided by Davidson *et al.* (1990). In this study, recombinant human plasminogen was synthesized in Sf9 cells using a serum-free medium (EX-CELL 400, see Chapter 5). Analysis of the asparagine-linked oligosaccharides revealed that 60% of the side-chains were of the high-mannose type, as described above. The remaining 40% were of a more complex type consisting primarily of ($GlcNAc_2$-Man-(Man-GlcNAc-Gal-Sia)$_2$), indicating that mannosidase, galactosyl-hexaminidasyl- and sialyl-transferases were present or could be induced in cultured insect cells.

Another report has demonstrated that insect cells are able to perform O-linked glycosylation (Thomsen *et al.*, 1990). In this study pseudorabies gp50, which has no consensus N-linked glycosylation sites, was shown to be glycosylated by radiolabelling infected cells with [^{14}C]glucosamine. Further analyses demonstrated the nature of the oligosaccharide side-chains to be mainly GalNAc, with lower amounts of Galβ1-3GalNAc; no sialic acid residues were detected. Measurement of the relevant glycosyltransferases revealed that Sf9 cells contained similar levels of UDP-GalNAc, N-acetylgalactosaminyltransferases as Vero and CHO cells, but lower levels of UDP-Gal:N-acetylgalactosamine-β1-3galactosyltransferase activity.

Where assays of biological activity have been carried out, glycoproteins synthesized in insect cells have been shown to have similar, if not identical, activities to their authentic counterparts. For example, influenza virus, fowl-plague virus (FPV), and parainfluenza virus (type 3) haemagglutinins (HA) were able to haemagglutinate red blood cells (Kuroda *et al.*, 1986; Possee, 1986; Van Wyke Coelingh *et al.*, 1987). Several other virus glycoproteins have been expressed in insect cells and all have either been membrane targeted or secreted, as appropriate; some examples of these have been included in Table 3.1. In each of these examples the glycoproteins synthesized were antigenic, giving rise to high-titre antibody preparations after injection into animals. However, not all the antibodies raised were neutralizing or gave protection against challenge by the live virus; whether this may be attributed to differences in glycosylation is debatable.

Both human t-PA (Jarvis and Summers, 1989) and urinary-type plasminogen activator (u-PA; King *et al.*, 1991b) have been secreted in active forms. The baculovirus expressed u-PA was shown to dissolve fibrin clots in both fibrin-well assays and by using zymography. Injection of juvenile hormone esterase, secreted from insect cells infected with a recombinant baculovirus, into *Manduca sexta* larvae caused the caterpillars to turn black (melanize) at the next moult (Hammock *et al.*, 1990). Melanization is a common assay for anti-juvenile hormone activity.

Many glycoproteins have been correctly targeted and inserted into the insect cell plasma membrane. These include the three HA proteins described above and also the human epidermal growth factor (EGF) receptor (Greenfield *et al.*, 1988), the human insulin receptor (Herrera *et al.*, 1988; Paul *et al.*, 1990), the human CD4 receptor (Webb *et al.*, 1989) and the chick nicotinic acetylcholine receptor α-subunit (Atkinson *et al.*, 1990b). This topic is discussed in more detail in section 3.5.

In summary, there are differences in glycosylation between insect and other cell types. Whether this has a bearing on the biological activity or antigenicity of any given glycoprotein will probably have to be tested empirically for each example. There are some concerns that differences in glycosylation may affect the usefulness of potential therapeutic proteins made in insect cells. For example, concern has been expressed about differences in antigenicity (Anicetti *et al.*, 1989; Marino, 1989) and in clearance rates from plasma, since one study has shown that glycoproteins with oligosaccharides terminating in mannose, GlcNAc or galactose are rapidly cleared from plasma (Stahl, 1990). There is some preliminary evidence that different types of insect cells (derived from different species or tissue types) may have alternative glycosylation pathways to those described above for *S. frugiperda* cells (Klenk, H. D., personal communication) and these may prove to be useful for glycoprotein synthesis. In the future, it may also prove possible to engineer insect cell lines to encode mammalian oligosaccharide transferases and thus complete the trimming and processing of the core oligosaccharide.

3.3 PHOSPHORYLATION, ACYLATION AND AMIDATION

Phosphorylation of a number of recombinant proteins expressed in insect cells have been reported and examples of these are listed in Table 3.2. As will be described in section 3.5, many of the recombinant proteins that have been shown to be targeted to the nucleus are phosphorylated. The combined data from these reports

Table 3.2 Examples of foreign proteins that have been phosphorylated, acylated or amidated in insect cells

Protein	*Species/virus*	*Phosphorylated (P) Acylated (Ac)*[1] *Amidated (Am)*	*Reference*
Virus examples			
E1A	Adenovirus	P	Patel *et al.* (1988)
E2 protein	Bovine papillomavirus (type 1)	P	McBride *et al.* (1989)
Core antigen	Hepatitis B virus	P	Lanford and Notvall (1990)
Surface antigen	Hepatitis B virus	Ac-M	Lanford *et al.* (1989)
$p17^{gag}$	HIV	Ac-M	Overton *et al.* (1989)
$p24^{gag}$	HIV	P	Overton *et al.* (1989)
$p40^{x}$ trans-activator	Human T-cell leukaemia virus (HTLV- I)	P	Jeang *et al.* (1987a) Nyunoya *et al.* (1988)
Nucleoprotein	Rabies virus	P	Préhaud *et al.* (1990)
Large T antigen	SV40 virus	P, Ac-P	Lanford (1988) Murphy *et al.* (1988)
gag precursor	Simian immuno-deficiency virus (SIV)	Ac-M	Delchambre *et al.* (1989)
Non-virus examples			
Krüppel	*Drosophila*	P	Ollo and Maniatis (1987)
Diuretic hormone	*M. sexta*	Am	Maeda (1989b)
EGF receptor	Human	P	Greenfield *et al.* (1988)
Insulin receptor	Human	P	Herrera *et al.* (1988) Paul *et al.* (1990)
c-*myc* proto oncogene	Human	P	Miyamoto *et al.* (1985)
$pp60^{c\text{-}src}$ oncogene	Human	P,Ac-M	Piwnica-Worms *et al.* (1990)
P^{210} BCR-ABL oncogene	Human	P	Pendergast *et al.* (1989)
Terminal transferase	Human	P	Chang *et al.* (1988)
Transferrin receptor	Human	Ac-P	Domingo and Trowbridge (1988)
Protein kinase C-γ	Bovine	P	Patel *et al.* (1988, 1989)
p53	Murine	P	O'Reilly and Miller (1988)
Tyrosine hydroxylase	Rat	P	Fitzpatrick *et al.* (1989)
$pp60^{v\text{-}src}$ (rsk- α/β)	*Xenopus*	P	Vik *et al.* (1990)
Transposon Ac	*Zea mays* (corn)	P	Hauser *et al.* (1988)

[1] Ac-M = myristylation. Ac-P = palmitylation.

suggest that phosphorylation is carried out by an endogenous protein kinase, with the major phosphoamino acid being phosphoserine (Ellis *et al.*, 1988; Nyunoya *et al.*, 1988). The fidelity of phosphorylation in insect cells has been demonstrated by the autophosphorylation of a number of recombinant proteins, including the human EGF receptor (Greenfield *et al.*, 1988), the human insulin receptor (Herrera *et al.*, 1988; Paul *et al.*, 1990) and the P^{210} BCR-ABL oncogene product, associated with chronic myelogenous leukaemia (Pendergast *et al.*, 1989). One recent study has shown that the $pp60^{c\text{-}src}$ oncogene product is phosphorylated at tyr 416, and that this activated kinase can then phosphorylate co-expressed polyoma virus middle T antigen (Piwnica-Worms *et al.*, 1990). Overton *et al.* (1989) have demonstrated that human immunodeficiency virus (HIV) $p55^{gag}$ is processed to give p24 and p17 when co- expressed with HIV protease. The p24 gene product is subsequently phosphorylated and the p17 gene product is acylated.

At least two types of fatty acid acylation have been demonstrated in insect cells: palmitylation and myristylation. The SV40 large T antigen has been labelled with [^{3}H] palmitic acid, a normal processing requirement for the membrane-targeting of this viral protein (Lanford, 1988). The hepatitis B virus surface antigen (Lanford *et al.*, 1989) and the HIV gag p17 gene product (Overton *et al.*, 1989) are acylated with myristic acid at the amino-terminus.

To date no amidation activity has been demonstrated in the processing of mammalian or virus proteins in insect cells *in vitro*, although it is well documented that insect larvae produce hormones that are amidated, for example, the diuretic hormone of *M. sexta* (Kataoka *et al.*, 1989). When this diuretic hormone was synthesized using a baculovirus vector, it was found to be amidated at the C-terminus (Maeda, 1989b). One study by Lebacq-Verheyden *et al.* (1988) has shown that, although the gastrin-releasing peptide precursor is cleaved correctly in insect cells, the processed peptide is not amidated and consequently is not fully active.

3.4 PROTEOLYTIC PROCESSING

Insect cell proteolytic enzymes appear capable of recognizing and accurately cleaving a number of recombinant proteins. This section will focus on proteolytic processing events excluding cleavage of signal peptides, which will be discussed in section 3.5. Trypsin-like endoprotease and carboxypeptidase N activities in insect cells have been inferred from the correct maturation of the FPV HA (Kuroda *et al.*, 1986, 1989). In these studies haemolytic fusion activity of

FPV HA, which is dependent upon cleavage of HA into HA1 and HA2 subunits, was demonstrated. In another example, the correct processing of human respiratory syncytial virus (RSV) F glycoprotein was demonstrated. The precursor F0 (68 kDa) was cleaved to give two subunits, F1 (48 kDa) and F2 (20 kDa), which were held together by disulphide bonds (Wathen *et al.*, 1989).

Two other studies, however, did not detect endoproteolytic cleavages. The HA of the A/PR/8/34 strain of influenza virus was not processed to HA1 and HA2 (Possee, 1986) and the HIV gp160 was not cleaved into gp120 and gp41 (Rusche *et al.*, 1987). In a more recent study, HIV p55gag was shown to be processed correctly, to give p17 and p24, only when co-expressed with the HIV protease gene (Overton *et al.*, 1989).

It would therefore appear that some viral proteins are correctly cleaved in insect cells, whereas, others require concomitant expression of the necessary virus-encoded proteases. There are several examples of virus polyproteins, encoding viral proteases, that are correctly processed in insect cells. These include Sindbis virus (Oker-Blom and Summers, 1989), poliovirus type 3 (Urakawa *et al.*, 1989) and foot-and-mouth-disease virus (FMDV) (Roosien *et al.*, 1990). The poliovirus example is perhaps the most striking. In this study, the 6.6 kb coding region of poliovirus type 3 was expressed in insect cells and produced non-infectious, empty poliovirus capsids (Urakawa *et al.*, 1989). The capsids had the configuration VP0, VP1 and VP3. Thus the insect cells had permitted all the complex processing events in the maturation of the poliovirus virion, with the exception of the final cleavage of VP0 to VP2 and VP4, which is auto-catalytic upon encapsidation of the virus RNA genome.

3.5 CELLULAR TARGETING AND SECRETION

Proteins synthesized by baculovirus vectors appear to be translocated in the insect cell as would be expected for the normal protein in its own host cell. For example, most signal peptides are recognized and cleaved in the endoplasmic reticulum and the resulting protein is targeted to the membrane or secreted, as appropriate. Amino-terminal sequence analysis has confirmed the correct signal peptide cleavage for human α-interferon (Maeda *et al.*, 1985), human interleukin-2 (IL2; Smith *et al.*, 1985), juvenile hormone esterase (Hammock *et al.*, 1990) and the ALV env gp85 (Noteborn *et al.*, 1990). In one detailed study, Lebacq-Verheyden *et al.* (1988) demonstrated that human gastrin-releasing peptide precursor (GRP1-125) was correctly cleaved to give an active peptide, GRP1-27 and a C-terminal portion

GRP31-125. However, a number of other novel cleavage forms were also identified that were not found when GRP was processed in a mammalian lung cancer cell line.

Membrane targeting also depends on the presence of a hydrophobic, membrane anchor sequence. Deletion of this region may enable the protein to be secreted. For example, the *Torpedo* nicotinic acetylcholine receptor α-subunit is secreted from insect cells if the membrane anchor region is deleted (Atkinson, A.E., Akhtar, R., Barkas, T. and King, L.A., unpublished data). Secretion of proteins has particular advantages in the purification of gene products. As with other expression systems, however, it is probably not always sufficient simply to add a signal peptide to a protein that is not normally secreted and expect it to translocate to the culture medium. The nature of the foreign protein may preclude its passage through membranes.

Examples of recombinant glycoproteins that have been efficiently secreted or membrane-targeted are listed in Table 3.1. A number of other recombinant proteins that appear not to have been glycosylated have also been efficiently secreted from insect cells, including: ricin (from *Ricinus communis*, castor bean; Piatak *et al.*, 1988); the human immune activation protein, act-2 (Lipes *et al.*, 1988); the extracellular domain of nerve growth factor receptor (Vissavajjhala and Ross, 1990) and platelet-derived growth factor (Giese *et al.*, 1989).

Baculovirus-expressed proteins may also be found in the cytoplasm or nucleus. Many of the proteins targeted to the nucleus are phosphorylated (see section 3.3), for example, the c-*myc* gene product (Miyamoto *et al.*, 1985), the *Drosophila* Krüppel gene product (Ollo and Maniatis, 1987) and the SV40 and polyoma virus large T antigens (Rice *et al.*, 1987; Murphy *et al.*, 1988). Others are located in the nucleus without phosphorylation, for example, Dengue virus core protein (Makino *et al.*, 1989). It still remains to be conclusively demonstrated that insect cells use the same nuclear transfer signals as mammalian cells.

Proteins located in the cytoplasm may form insoluble inclusion bodies, such as the lymphocytic choriomeningitis virus N protein (Matsuura *et al.*, 1987) and the Lassa fever virus nucleocapsid protein (Barber *et al.*, 1990), or may be completely soluble such as the human glucocorticoid receptor (Srinivasan and Thompson, 1990). Other recombinant proteins may be only partly soluble, for example, the catalytic subunit of protein phosphatase 1, a key enzyme in the regulation of many cellular functions (Berndt and Cohen, 1990). This protein was synthesized to high levels (25% total cell protein) in the cytoplasm where approximately 5% was found as a soluble, active species and the remaining 95% as an insoluble, inactive species. The

insoluble species could be dissolved in 6 M guanidinium chloride to give a fully active protein.

3.6 TERTIARY AND QUATERNARY STRUCTURE FORMATION

Perhaps one of the most useful features of the baculovirus system is the ability of the recombinant proteins to form tertiary and complex quaternary structures. In particular, the multiple expression capabilities of the system allow two or more proteins to be synthesized simultaneously and thus any interactions forming between the proteins can be analysed (see Chapter 2). The formation of a complex between two of the three influenza virus polymerase proteins (St Angelo *et al.*, 1987), between the SV40 large T antigen and human p53 proteins (O'Reilly and Miller, 1988), and between the HIV gp120 and the soluble domains of the CD4 (SCD4) receptor (Morikawa *et al.*, 1990) have been demonstrated. Co-infection of insect cells with three recombinant viruses expressing the UL5, UL8 and UL52 genes of herpes simplex virus (HSV) type 1 produced a functional helicase–primase complex (Dodson *et al.*, 1989). This complex consisted of three polypeptide chains with a combined molecular weight of 270 kDa and was shown to have authentic DNA-dependent ATPase and GTPase, DNA helicase and DNA primase activities.

The formation of discrete sub-viral particles has been observed for the hepatitis B virus (HBV) surface (s) and core (c) antigens (Kang *et al.*, 1987; Takehara *et al.*, 1988; Lanford *et al.*, 1989). Kang *et al.* (1987) demonstrated the accumulation of 22 nm particles in the culture medium of insect cells expressing the HBVs antigen. These particles appeared to be identical to the 22 nm particles found in the plasma of patients with chronic hepatitis. Co-expression of the HBV s and c antigens gave rise to 27 nm particles (Takehara *et al.*, 1988).

In insect cells infected with recombinant viruses expressing the bluetongue virus (BTV) 10 NS1 gene product, tubule structures were formed similar to those observed in mammalian cells infected with BTV (Urakawa and Roy, 1988). Further studies on the morphogenesis of bluetongue virus have been carried out by making use of baculovirus multiple expression vectors. Core-like structures were observed when the two major BTV core proteins were co-expressed (French and Roy, 1990) and a double-shelled, virus-like particle was assembled when four BTV proteins were expressed simultaneously (French *et al.*, 1990). In this example, multiple expression was achieved by the co-infection of cells with two recombinant viruses, each synthesizing two BTV proteins (see Chapter 2 for further details on multiple expression vectors). As already mentioned, expression

of the entire coding region of poliovirus type 3 gives rise to the assembly of non-infectious, intact virions containing VP0, VP1 and VP3 (Urakawa *et al.*, 1989). When the poliovirus capsid coding region alone was expressed, the only protein synthesized was an uncleaved precursor, thus demonstrating the requirement for the poliovirus encoded protease to complete the proteolytic processing events (Urakawa et al., 1989).

Although most of the studies on quaternary structure formation have focused on virus proteins, there are also examples of other recombinant proteins that have formed complex structures in insect cells. These include: dimeric assembly of the human macrophage-colony-stimulating factor (Maiorella *et al.*, 1988) and platelet-derived growth factor (Giese *et al.*, 1989); heterodimer (α and β chain) formation of the extracellular domain of the insulin receptor (Sissom and Ellis, 1989); dimeric assembly of the extracellular domain of the nerve growth factor receptor (Vissavajjhala and Ross, 1990) and the transferrin receptor (Domingo and Trowbridge, 1988); and tetrameric assembly of rat tyrosine hydroxylase (Forstová *et al.*, 1989).

3.7 EXPRESSION OF VIRAL GENES

The baculovirus system has been used extensively to express virus structural and non-structural genes. Some of these have been used as examples in the sections covered above, for example, virus glycoproteins (section 3.2). Many of the virus structural proteins have been synthesized to very high levels and virtually all published examples have proved to be antigenic, giving rise to high-titre antisera. In many cases challenge experiments with live virus have shown that the insect cell-derived proteins are capable of inducing protective, neutralizing antibodies. Examples of virus structural and non-structural proteins synthesized in insect cells are given in Table 3.3, except glycoproteins which have been included in Table 3.1.

3.8 EXPRESSION OF BACTERIAL AND FUNGAL GENES

The sections above refer almost exclusively to the expression of eukaryotic or virus genes. A number of bacterial and fungal genes have also been expressed very successfully in insect cells. Examples include: *Escherichia coli* β-galactosidase (Pennock *et al.*, 1984; and subsequently by many workers), β-glucuronidase (Luckow and Summers, 1989) and chloramphenicol acetyltransferase (Luckow and Summers, 1988b, 1989); *Bacillus thuringiensis* (subspecies *Kurstaki* HD73) delta-endotoxin (Martens *et al.*, 1990; Merryweather *et al.*, 1990); and *B. anthracis* protective antigen (Iacono-Conners *et al.*,

Table 3.3 Examples of virus structural and non-structural proteins synthesized in insect cells[1]

Protein	*Virus*	*Structural (S), Non-structural (NS)*	*Reference*
DNA polymerase	Adenovirus type 2	NS	Watson and Hay (1990)
VP7 (core)	Bluetongue virus (type 10)	S	Oldfield *et al.* (1990)
NS3 and NS3A	Bluetongue virus (type 10)	NS	French *et al.* (1989)
Four proteins	Bluetongue virus (type 10)	S[2]	French *et al.* (1990)
Two core proteins	Bluetongue virus (type 10)	S[2]	French and Roy (1990)
NS1 gene	Bluetongue virus (type 10)	NS[2]	Urakawa and Roy (1988)
E2 ORF	Bovine papillomavirus	NS	Tada *et al.* (1988)
I gene	Cauliflower mosaic virus	NS	Vlak *et al.* (1990)
Core	Dengue virus	S	Makino *et al.* (1989)
gag proteins	Feline immunodeficiency virus	S[2]	Morikawa *et al.* (1991)
Polyprotein	Foot-and-mouth-disease-virus	NS, S	Roosien *et al.* (1990)
Capsid proteins	Hepatitis A virus	S	Harmon *et al.* (1988)
Core antigen	Hepatitis B virus	S[2]	Takehara *et al.* (1988) Hilditch *et al.* (1990)
DNA polymerase	Herpes simplex virus	NS	Marcy *et al.* (1990)
Helicase–primase	Herpes simplex virus (type 1)	NS	Dodson *et al.* (1989)
Protease	HIV	NS	Overton *et al.* (1989)
$p17^{gag}$, $p24^{gag}$	HIV	S	Overton *et al.* (1989)
gag	HIV	S	Madisen *et al.* (1987)
Nucleoproteins	Influenza A and B viruses	S	Rota *et al.* (1990)
Three polymerase proteins	Influenza virus	NS	St Angelo *et al.* (1987)
VP1 and VP2	Parvovirus (human)	S[2]	Brown *et al.* (1991)
Polyprotein	Poliovirus (type 3)	NS, S[2]	Urakawa *et al.* (1989)
Large T antigen	Polyoma virus	NS	Rice *et al.* (1987)
Nucleoprotein	Rabies virus	S	Préhaud *et al.* (1990)
VP4 (outer capsid)	Rotavirus	S	Nishikawa *et al.* (1989)
Major capsid (VP6)	Rotavirus	S[2]	Estes *et al.* (1987)
Polyprotein	Sindbis virus	NS, S	Oker-Blom and Summers (1989)
Nucleoprotein	Snowshoe hare bunyavirus	S	Urakawa *et al.* (1988)
NSs	Snowshoe hare bunyavirus	NS	Urakawa *et al.* (1988)
Large T antigen	SV40	NS	Lanford (1988) O'Reilly and Miller (1988)
Small t antigen	SV40	NS	Jeang *et al.* (1987b)

[1] Glycoproteins are included in Table 3.1.
[2] Tertiary or quaternary structures formed.

1990). Finally, the fungal qa-1F activator protein from *Neurospora crassa* has been successfully synthesized in insect cells, where other systems (bacterial, yeast) have failed due to the cytotoxic nature of this protein (Baum *et al.*, 1987).

3.9 POST-TRANSCRIPTIONAL PROCESSING

There is very little published information concerning the ability of the baculovirus system to cope with the expression of intron-containing genes. Other processing events such as polyadenylation and 5′ capping of mRNAs are known to occur. One report has tentatively concluded that the insect cell can distinguish splice acceptor–donor sites. In this study, a segment of SV40 genomic DNA encoding the large T and small t antigens was expressed in a baculovirus vector (Jeang *et al.*, 1987b). Only small t antigen was detected in the insect cells and the authors concluded that this demonstrated that the splice site for small t antigen was preferentially utilized. To date, there have been no published reports that an intron-containing gene can be accurately spliced to give the correct mRNA for faithful translation.